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Title	Reactive Oxygen Species and Low-Dose Effects of Tritium on Bacterial Cells
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Abstract

The paper continues study of exposures of luminous marine bacteria to low-dose radiation of tritium; tritiated water (HTO) was applied as a source of the irradiation. Hypothesis on involvement of Reactive Oxygen Species (ROS) to signaling mechanism of bacterial cells under exposure to low-intensity tritium radiation was verified. Bacterial bioluminescence intensity was considered as a tested physiological parameter; it was compared to the ROS production in the bacterial environment of different activity concentrations: 0.03, 4.0, and 500 MBq/L. Exposure of the bacteria to chronic low-dose tritium irradiation (<0.08 Gy) increased bioluminescence intensity and ROS production considerably (up to 300%). Spearman rank correlation coefficients were calculated and confirmed the relations between bioluminescence intensity and ROS production. Additional peculiarities of HTO effect were: independence of bioluminescence intensity and ROS content on HTO activity concentration; low ROS content in bacteria-free aquatic environment. Effects of HTO on bacterial bioluminescence were attributed to: (1) trigger function of products of tritium decay for bacterial metabolic oxygen-dependent processes, with bioluminescence involved; (2) signaling role of ROS as intercellular messengers in “bystander effect”; (3) fixed amount of bacterial cells (3 107 cells per milliliter) provided the upper limits of the bioluminescence intensity and ROS content. As an outlook, in spite of low energy of tritium decay, its influence on aquatic biota via ROS production by microorganisms should be taken into consideration.

Keywords	Tritium; low-dose effect; luminous marine bacterium; Reactive Oxygen Species, radiation hormesis, bystander effect, signal molecules
Taxonomy	Environmental Radioactivity, Biomonitoring, Environmental Monitoring, Water Pollution

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Dear Sir,

We would like to thank reviewers for the interest and attention to our manuscript, as well as for useful remarks.

We forward you our correction according to remarks of reviewers.

The file with corrections marked with color is attached.

Reviewer 1

1). Sub-section 2.2 – The authors did not give the data regarding the bioluminescence kinetics. They are not provided anywhere throughout the manuscript.

Reply:

The following phrase was added and marked in the manuscript:

Sub-section 2.2 – fifth paragraph – "The bioluminescence kinetics measurements went on for 52 h until the bioluminescent intensity in the control samples decreased to 20% of the initial one. Measurements of the bioluminescence intensity were carried out using standard procedure (Kuznetsov et al., 1996; Kudryasheva et al., 1998)."

2). The ROS species (O_2^-, H_2O_2, HO°) are obtained in the experiment by the dosage of H_2O_2, but the dosage of O_2^- and HO° is not carried out and is important. Besides, the correlation factors between concentrations of O_2^- and HO° and concentration of H_2O_2 are not given.

Reply:

To evaluate changes of ROS content we used chemiluminescence luminol method. It is based on measuring the chemiluminescence intensity which can be considered as a rate of the chemiluminescent reaction, being proportional to the ROS concentration. The aim of our study is concerned with evaluation of the CHANGES of ROS content under variation of external conditions (HTO concentration, time of exposure in bacterial or bacteria-free media), and the luminol reaction is just a proper tool for this evaluation due to simplicity and high rate of luminescence registration. This method evaluates integral content of ROS in relative units, and this is sufficient to follow the aim of the current study. Absolute values of different ROS species in

solution is a very delicate question, in biological (not model) systems particularly. Balance of the different ROS species is variable; it depends on a lot of factors (internal and external) and requires a special consideration. This can be interesting continuation of the current work with another purpose and additional experimental methods. Additionally, it should be noted that application of spectral methods to study ROS species in biological systems (not model) can be problematical.

3). Internally, ROS species are produced inside the bacteria, by the interaction of HOT with the molecular structures existed in the bacteria. Externally, the ROS species are produced in the exterior of the bacteria by the interaction with HTO.

Reply:

Really, mechanisms of low-dose effects are not simple. Evidences for this are the following: (a) Previously, we showed that tritium can activate bacterial bioluminescence without its penetration to the bacterial cells; these results were concerned with "intensification of trans-membrane cellular processes stimulated by ionization and radiolysis of aqueous media" (Rozhko et al., 2016) (Section 3.1 – paragraph 3); (b) addition of tritiated water does not increase ROS in water solutions noticeably – this is discussed below (remark 4).

4). Sub-section 3.1 – fifth paragraph – "Hence, ROS, which are constantly produced by the bacterial cells in the processes of their aerobic functioning, are proper candidates for intercellular messengers in the lowintensity tritium environment." ROS can be produced not only by the bacterial cells, but in the water environment where the bacteria live, also.

Reply:

It is very important point. We supposed the same previously. However, we confirmed previous results (Selivanova et al., 2013) and showed in this study, that addition of tritiated water does not increase ROS in water solutions.

We added the following phases:

Sub-section 3.1 – fifth paragraph - **"**Since low-intensity tritium exposure does not increase noticeably the ROS content in bacteria-free media (Selivanova et al., 2014), non-biological production of ROS can be hardly responsible for this effect.

This aspect of ROS production will be discussed in the next sections."

Sub-section 3.2 – third paragraph – **"**Figure 2 demonstrates that addition of HTO to 3% NaCl solution did not increase amount of ROS at three HTO radioactivities used (0.03, 4, and 500 MBq/L); the ROS content was low in all radioactive and non-radioactive NaCl solutions (compare Fig.2A and Fig.2B)."

We pay attention to the following positions:

Sub-section 3.3 – third paragraph – "Since the rates of ROS production in bacteria-free HTO are low…."

Sub-section 4. Conclusion– last paragraph – "The mechanism of tritium influence on aquatic biota can be concerned with secondary processes, i.e. production of excess amount of ROS by microorganisms due to intensification of their cellular metabolic processes."

5). Fig. 2 – The concentration of ROS species is given as 10 – 30 x 10^{7} M. Please, correct. It must be 10 – 30 x 10^{-7} M.

Reply: We corrected Axis title in Fig.2.

Reviewer 2

Introduction: Composition of ROS group should be proper to consider just after first mentioning.

Reply: Corrected.

The following phrases should be corrected:

Section 3.1.

'Activation of the bacterial bioluminescence by tritium was demonstrated in several experiments.' Instead of: 'Activation of the bacterial bioluminescence by tritium was demonstrated in many experiments'.

Reply: Corrected.

‘Such a low value of par-ions per cell suggests a specific mechanism of tritium influence on the cells.’ Instead of: ‘Possibility of such a low value of par-ions per cell suggests a specific mechanism of tritium influence on the cells.’

Reply: Corrected.

‘Probably, products of tritium decay can function as “triggers” of intensification of metabolic oxygen-dependent processes in several cells and intracellular ROS production. ‘Instead of: ‘Probably, products of tritium decay can be considered as “triggers” for intensification of metabolic oxygen-dependent processes in several cells and intracellular ROS production.

Reply: Corrected.

Section 3.3.

‘…so we can suppose that the same probabilistic character processes are responsible for..’Instead of: ‘…so we can suppose that processes of the same probabilistic character are responsible for…’

Reply: Corrected.

‘Trigger function of products of tritium decay in bacterial metabolic oxygen-dependent processes,’ Instead of: ‘Trigger function of products of tritium decay for bacterial metabolic oxygen-dependent processes.’

Reply: Corrected.

ROS content in bacteria-free tritiated water is low

Increase of bacterial luminescence and ROS content were found in tritiated water

Bacterial luminescence intensity and ROS production in tritiated water are related

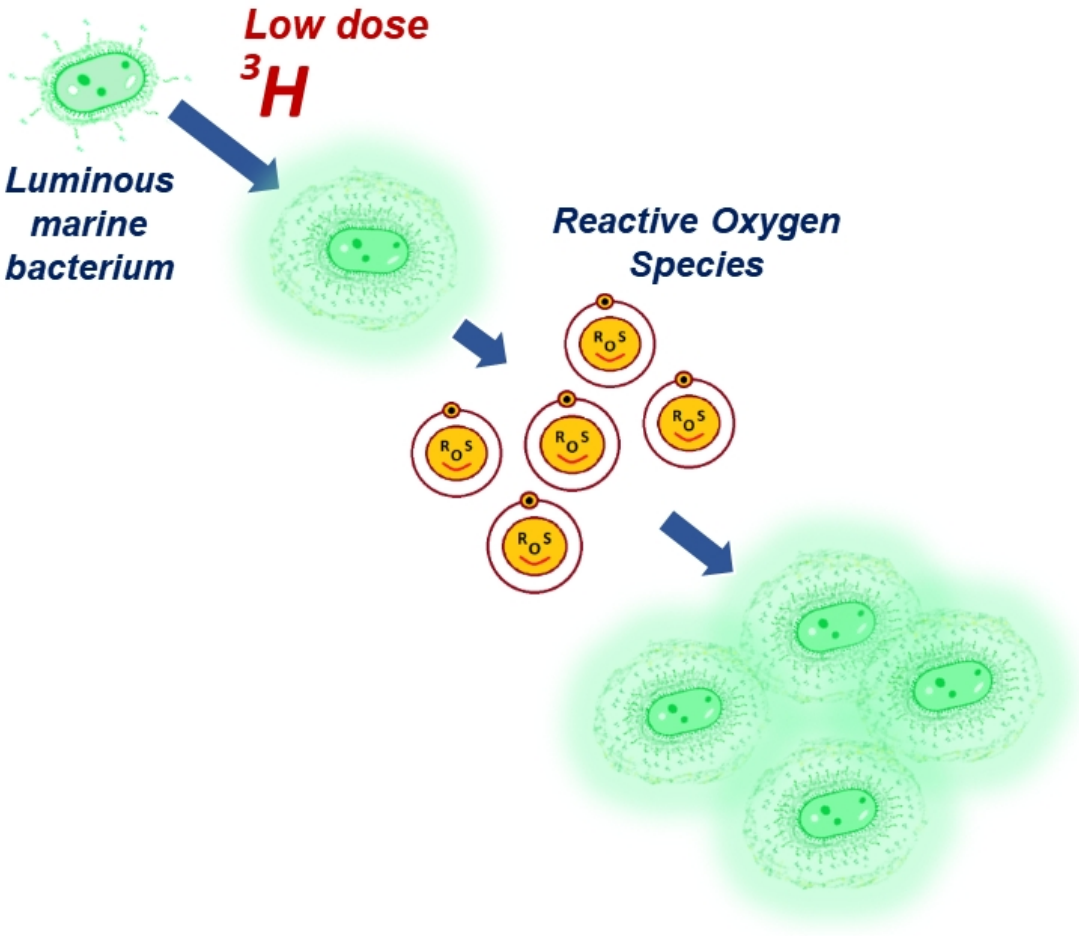
Bioluminescence and ROS content don’t depend on tritium activity concentration

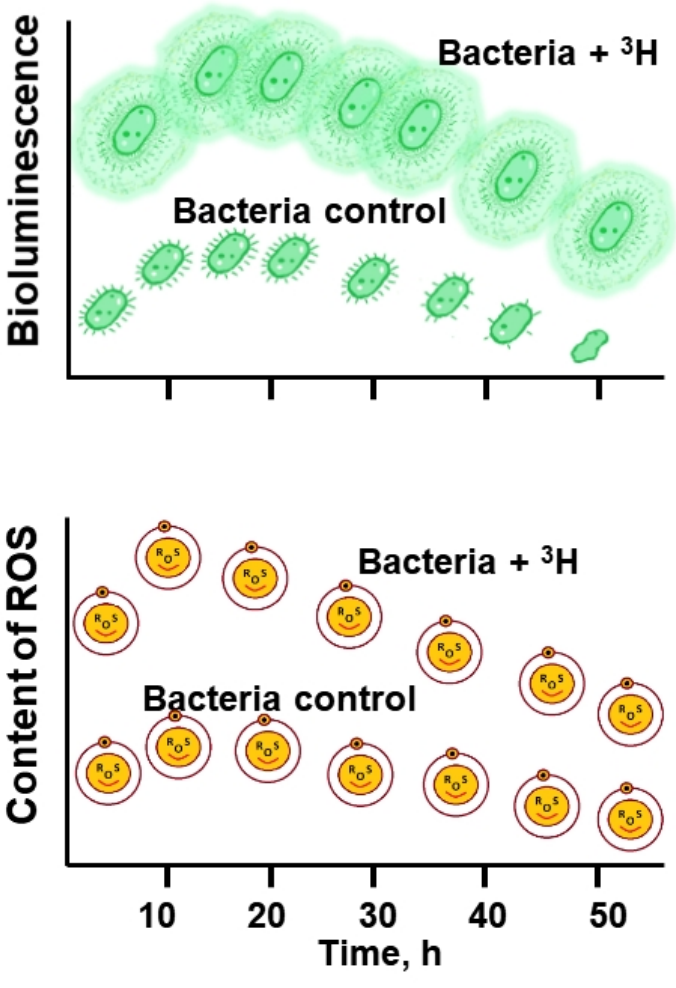
ROS are signaling particles in bystander effect for bacterial aqueous suspensions

ABSTRACT

The paper continues study of exposures of luminous marine bacteria to low-dose radiation of tritium; tritiated water (HTO) was applied as a source of the irradiation. Hypothesis on involvement of Reactive Oxygen Species (ROS) to signaling mechanism of bacterial cells under exposure to low-intensity tritium radiation was verified. Bacterial bioluminescence intensity was considered as a tested physiological parameter; it was compared to the ROS production in the bacterial environment of different activity concentrations: 0.03, 4.0, and 500 MBq/L. Exposure of the bacteria to chronic low-dose tritium irradiation (<0.08 Gy) increased bioluminescence intensity and ROS production considerably (up to 300%). Spearman rank correlation coefficients were calculated and confirmed relations between the bioluminescence intensity and ROS production. Additional peculiarities of HTO effect were: independence of the bioluminescence intensity and ROS content on HTO activity concentration; low ROS content in bacteria-free aquatic environment. Effects of HTO on bacterial bioluminescence were attributed to: (1) trigger function of tritium decay products in the bacterial metabolic oxygen-dependent processes, with bioluminescence involved; (2) signaling role of ROS as intercellular messengers in "bystander effect"; (3) fixed amount of bacterial cells ($3 \cdot 10^7$ cells/mL) provided an upper limits of the bioluminescence intensity and ROS content. As an outlook, in spite of low energy of tritium decay, its influence on aquatic biota via ROS production by microorganisms should be taken into consideration.

Keywords: tritium, low-dose effect, luminous marine bacterium, reactive oxygen species, radiation hormesis, bystander effect, signaling molecules


Low dose
^3H
Luminous marine bacterium
Reactive Oxygen Species
ROS


Bioluminescence
Bacteria + ^3H
Bacteria control
Content of ROS
Bacteria + ^3H
Bacteria control
10
20
30
40
50
Time, h

Reactive Oxygen Species and Low-Dose Effects of Tritium on Bacterial Cells

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1. Introduction

Biological effects of low-dose exposures are currently of special interest due to expansion of areas with low-intensity radioactive contaminations. Products of radioactive decay of the contaminants can impact on conjugated chemical and biological processes in natural ecosystems, resulting in changes of natural balance in ecosystem overall.

Biological effects of low-dose radiation are currently associated with production of Reactive Oxygen Species (ROS) (Matsumoto et al., 2007; Smith et al., 2013). It is known that radionuclides produce ROS in water solutions (Selivanova et al., 2014; Alexandrova et al., 2011; Azzam et al., 2012) as a result of water radiolysis in the presence of dissolved molecular oxygen. On the other part, ROS are products of natural metabolism of aquatic organisms (Brynildsen et al., 2013; Ezraty et al., 2017). Complex interrelations take place between the ROS production and changes of physiological functions of organisms in radioactive solutions. Low-dose irradiation of organisms is of special interest, as it can activate physiological functions, along with their suppression – effect of "radiation hormesis" (Calabrese, 2018; Jargin, 2018; Shibamoto and Nakamura, 2018).

ROS are unstable highly-reactive compounds, products of incomplete oxygen reduction; they are continuously generated, changed and consumed in all living organisms as a result of their aerobic functioning. Group of ROS includes superoxide anion (O_2•−), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•) and others (Ezraty et al., 2017). Traditionally, ROS are considered as initiators of oxidative stress and damage, which lead to deterioration of tissues and organs, aging and diseases (Aprioku, 2013; Hybertson et al., 2011; Ezraty et al., 2017; Imlay, 2013). Nevertheless, a lot of studies do not support this hypothesis for organisms exposed to low-dose chronic radiation, typical of contaminated environments (Smith, et al., 2012). Data exist on positive ROS contribution to physiology. It has been shown that at low and moderate doses, ROS contribute to the regulation of vital physiological functions (Winterbourn, 2008; Alfadda and Sallam, 2012); they are responsible for proliferation, migration, differentiation, and metabolism (Griendling et al., 2016; Suzen et al., 2017). It should be noted that ROS serve as both intra- and intercellular messengers (Hancock et al., 2001; Kashmiri and Mankar, 2014) and initiate a cellular protective response (Matsumoto et al., 2007). The

radiation-induced bystander effect is elicited by agents and factors released by cells exposed to radiation (Sokolov and Neumann, 2018); reactive oxygen and nitrogen species are reasonable example of such agents. The study (Jella et al., 2018) demonstrates that the bystander effects can occur in cells exposed to media from irradiated cells. Chemistry and biology of ROS effects in signaling or stress responses is under consideration now (Dickinson and Chang, 2011; Zakhvataev, 2016).

Microorganisms are basic and simplest part of water ecosystems, contributing notably to ecosystem balance. Metabolic products of aquatic microorganisms may influence all water inhabitants. Marine luminous bacteria are proper bio-objects for study biological effects of radiation, with particular attention to their metabolites. In aerobic environments, bacteria form ROS endogenously through the reaction between O_2 and electron donors, such as metal centers, dihydroflavin, etc. It is known that peroxide derivatives of flavin molecule are native intermediate compounds of the bacterial bioluminescence reaction (Nemtseva and Kudryasheva, 2007; Lee et al., 2019). It was suggested that oxygen-detoxifying function provided the emergence of many bioluminescent systems, including bacterial bioluminescent one **(**Rees et al., 1998**).** This suggestion presents an additional argument to study ROS balance in radioactive bacterial environment.

Luminous marine bacteria have been used as a toxicity bioassay for several decades (Bulich and Isenberg, 1981; Abbas et al., 2018); mechanisms of toxic effects in bacteria are intensively studied (Kudryasheva, 2006; Kudryasheva and Rozhko, 2015). The tested parameter here is luminescence intensity; it can be easily measured instrumentally with simple physical devices. High rates of bioluminescence registration and simplicity of the test organism pave the way for simultaneous analyses of a lot of test-samples under comparable external conditions and a proper statistical processing. This advantage is very important for biological analyses, which are naturally characterized by lower reproducibility than chemical or radiometric assays.

Tritium is usually assumed as one of the less hazardous radioisotopes. Being one of the most widespread radioactive isotopes; tritium is incessantly generated in top layers of the Earth atmosphere as a result of space irradiation; the rate of its generation is 1200 atoms$\cdot s^{-1} \cdot m^{-2}$ (Lenskii, 1981). In the Earth, tritium is mostly presented as a component of tritiated water (HTO). Traces of HTO occur in

natural waters; its content was evaluated as one atom per 10^{18} hydrogen atoms. However, after nuclear tests in the 1950s, it increased. Tritium is a by-product of a lot of radiochemical processes in nuclear industry. Currently, concentration of HTO in the World Ocean varies; its local rise takes place around nuclear power plants and after nuclear incidents. These are reasons why low-dose effects of tritium are important for investigation now.

Tritium (T) is a beta-emitting radionuclide; its decay results in emission of electron and antineutrino:

$$T \overset{\beta^-}{\rightarrow} {}^{3}_{2}He^{+} + e^{-} + \tilde{\nu}$$

A half-life of tritium is 12.32 years; energy of beta decay is low (18.6 keV), average energy of electrons is 5,7 keV. Products of tritium decay (electron and ${}^{3}_{2}He^{+}$) are able to trigger charge/electron transfer chains in biochemical processes and affect a lot of charge-dependent processes in cells.

It is supposed that products of radioactive decay of HTO are electron and ROS:

$$H\text{–}O\text{–}T \rightarrow H\text{–}O\text{–}{}^{3}He^{+} + \bar{e}$$

$$H\text{–}O\text{–}{}^{3}He^{+} \rightarrow H\text{-}O^{\cdot +} + {}^{3}He$$

$$H\text{-}O^{+} + H_2O \rightarrow [O] + H_3O^{+},$$

where *[O]* is an atomic oxygen, representative of ROS. Water environment can stabilize free electrons (beta-particles) with hydrate complex formation.

Last decade, our group is involved to study the effects of tritium on luminous marine bacteria. Paper (Selivanova et al., 2013) considered an influence of tritiated water (0.0002-200 MBq/L) on the bacteria and their enzymatic reactions. Bioluminescent intensity, bacterial growth, cell damage, and tritium accumulation were under investigation (Selivanova et al., 2013; Kudryasheva and Rozhko, 2015).

Current paper studies interrelations between the bioluminescence intensity (as physiological parameter of luminous marine bacteria) and ROS production in bacterial aquatic environment under chronic low-dose exposure to tritium. The ROS content was evaluated by chemiluminescence method. Spearman correlation coefficients were calculated to confirm dependences between bioluminescence

intensity and ROS production. The ROS-dependent signaling mechanism of bacterial cells under exposure to low-intensity tritium radiation is under discussion.

2. Materials and methods

2.1. Reagents. Intact marine luminous bacterium, strain *Photobacterium phosphoreum* 1883 IBSO (Kuznetsov et al., 1996), was used. The *strain* was obtained from the Collection of Luminous Bacteria CCIBSO-836, Institute of Biophysics SB RAS, Krasnoyarsk, Russia. The NaCl preparation of analytical grade was applied to prepare the 3% solution for bacterial bioluminescence measurements.

The bacteria were cultivated at 22 °C, pH 7.2-7.4 on a semisynthetic nutrient medium (1 L distilled water, 30 g NaCl, 1g KH_2PO_4, 0.5 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 g $Na_2HPO_4 \cdot 12H_2O$, 3 g glycine, 5 g peptone).

Reagents for chemiluminescence measurements were the following: luminol from Sigma-Aldrich, hydrogen peroxide solution (H_2O_2) from Tula Pharmaceutical Factory, Russia, $K_3[Fe(CN)_6]$ from Khimreaktiv, Russia. The reagents were of chemical grade.

2.2. Bacterial bioluminescence measurements

The bacterial suspensions were exposed to low-dose radiation of tritium. Tritiated water, HTO, was used as a source of tritium. To imitate marine environment for bacterial cells and to balance osmotic processes, the 3% NaCl solutions were used. Bioluminescence kinetics of the bacterial samples was studied in tritium-free bacterial samples (control) and in HTO.

Control (non-radioactive) and radioactive bacterial suspensions were prepared as follows:

Radioactive samples: 10 µl of bacterial suspentions were added to 30 µl of the HTO in 3% NaCl solution. Specific HTO radioactivities in the bacterial suspensions were 0.03, 4, and 500 MBq/L.

Control samples: 10 µl of non-radioactive (control) suspentions were added to 30 µl of 3% NaCl solution.

To investigate chronic effects of the low-level beta-radiation of tritium on bacterial bioluminescence, radioactive and control samples of bacterial suspensions were maintained at +4 °C. The bioluminescence kinetics measurements went on for 52 h until the bioluminescent intensity in the

control samples decreased to 20% of the initial one. Measurements of the bioluminescence intensity were carried out using standard procedure (Kuznetsov et al., 1996; Kudryasheva et al., 1998). Bioluminescence intensities of control and radioactive samples were measured at room temperature and compared. Experimental error of the bioluminescence measurements did not exceed 1-2%.

Bioluminescence intensity was registered by Luminoskan Ascent (Thermal Fisher Corp.). All measurements were carried out at 20 °C.

2.3. Chemiluminescence measurements

To study ROS content in experimental solutions, the calibration dependence was preliminary determined as chemiluminescence intensity vs. H_2O_2 concentration. Concentration of aqueous alkaline luminol solution was 10^{-4}M. The chemiluminescence reaction was initiated by 10^{-3}M $K_3[Fe(CN)_6]$ solution.

Maximal chemiluminescence intensity was determined in 3% NaCl solutions and in bacterial suspensions (control and radioactive) after bioluminescence measurements. Luminol solution was added to the 3% NaCl solutions or bacterial samples. Chemiluminescence reaction was initiated by 75 μl solution of $K_3[Fe(CN)_6]$ through the injection system. Experimental error of the chemiluminescence measurements did not exceed 10%. Chemiluminescence intensity was used to calculate ROS content in the experimental solutions via the calibration dependence.

Chemiluminescence intensity was registered by Luminoskan Ascent (Thermal Fisher Corp.). All measurements were carried out at room temperature.

2.4. Statistical processing

To evaluate correlations between bioluminescence signal and ROS concentrations, statistical dependence between the rankings of two variables was analyzed (Gmurman et al., 1968), Spearman's rank correlation coefficients *r* were calculated. The application of this method was justified with a lack of normal distribution of bioluminescence intensity and ROS content, as well as moderate kit of data set.

3. Results and discussion

Earlier (Selivanova et al., 2013), a peculiar response of luminous marine bacteria to tritium (as a component of tritiated water, HTO) was revealed: absence of a monotonic dependence of luminescence response on tritium activity concentration was demonstrated under conditions of chronic low-dose exposures (< 0.03 Gy). The absence of monotonic dependence was found in a wide range of tritium concentrations (four orders), in suspensions of both intact and lyophilized bacteria. Basing on this result, we chose three activity concentrations of tritium in the four-order concentration range (0.03, 4, and 500 MBq/L) and compared time-courses of the bioluminescence response and ROS content in the bacterial suspensions. Maximum time of exposure to HTO corresponded to 0.08 Gy; this dose did not exceed a tentative low-dose limit.

3.1. Effect of tritium on biological luminescence of marine bacteria

Bacterial bioluminescence response to tritium exposure is shown in Figure 1. It is evident that HTO increases bioluminescence intensity about three times, as compared to the control bacteria sample. Inhibition of the bacterial bioluminescence was not observed in these experiments.

Detailed analysis of low-dose effect of tritium on luminescence of marine bacteria was conducted earlier (Selivanova et al., 2013; Kudryasheva and Rozhko, 2015). Luminescence was considered there as physiological parameter, along with bacterial growth (Alexandrova et al., 2010). All the effects of tritium corresponded to hormesis (i.e. non-linear) model of dose-effect dependence (Calabrese, 2018; Jargin, 2018; Shibamoto and Nakamura, 2018). Activation of the bacterial bioluminescence by tritium was demonstrated in several experiments. Bi-phasic time dependence (activation+ inhibition) was found in (Selivanova et al., 2013; Selivanova et al., 2014); and mono-phasic dependence (activation only) was found in (Rozhko et al., 2017; Rozhko et al., 2016). Current experiment (Fig.1) demonstrated mono-phasic dependence of bioluminescence intensity on time of exposure to tritium.

Probably, genetic mechanisms are not responsible for the bioluminescence activation due to the quick bacterial response to tritium. Additionally, sequence analysis did not reveal mutations in bacterial DNA under conditions of low-dose exposure to tritium (Rozhko et al., 2017); experiments with tritium-labeled films showed that activation of the bacterial bioluminescence can take place

without penetration of tritium to the bacterial cells; these results were concerned with "intensification of trans-membrane cellular processes stimulated by ionization and radiolysis of aqueous media" (Rozhko et al., 2016).

Fig.1 shows that the bioluminescence intensity was similar for three activity concentrations of tritium during all time of observation; with this confirming the results obtained earlier (Selivanova et al., 2013).

Independency of the bioluminescence intensity on tritium concentration requires a special consideration. Probably, bioluminescence intensity in HTO is upper-limited by low concentration of bacterial cells ($3 \cdot 10^7$ cells/mL). A special question is an interaction of tritium decay products with bacterial cells. Taking into consideration the concentration of cells and a number of par-ions in the tritium decay (up to 200 ions and/or excited molecules), we calculated the numbers of par-ions per cell: 3.33, 0.0266, 0.0002 par ions/(cell•s) for tritium activity concentrations 500000, 4000, 30 Bq/mL, respectively. Such a low value of par-ions per cell suggests a specific mechanism of tritium influence on the cells. Probably, products of tritium decay can function as "triggers" for intensification the metabolic oxygen-dependent processes in several cells, followed by the intracellular ROS production. These ROS being released to environment are supposed to serve as specific signaling molecules for other cells (so called "bystander effect"). Hence, ROS, which are constantly produced by the bacterial cells in the processes of their aerobic functioning, are proper candidates for intercellular messengers in the low-intensity tritium environment. Since low-intensity tritium exposure does not increase noticeably the ROS content in bacteria-free media (Selivanova et al., 2014), non-biological production of ROS can be hardly responsible for this effect. This aspect of ROS production will be discussed in the next sections.

It has been shown for human cells (Kadhim et al., 2004; Shao et al., 2003; Lyng et al., 2002) that the bystander effect can be induced by even one cell in a thousand-cell-population; it does not depend on a number of initially induced cells (one cell or 50% of cells).

Additionally, it should be paid attention that the absence of dose-response dependency is in accordance with a concept on "stochasticity" of low-dose radiobiological effects (Vasilenko and

Vasilenko, 2001). This concept assumes involvement of free radicals and ROS to the radiobiological responses.

To confirm the hypothesis on ROS involvement into the activation of the bacterial bioluminescence by tritium, the bioluminescence response (Fig.1) was compared to the ROS content in bacterial suspensions, as presented in the following Section.

3.2. Content of Reactive Oxygen Species in bacterial suspensions in HTO

ROS content was studied in control and radioactive bacterial suspensions. Preliminary, ROS content was studied in 3% NaCl bacteria-free solutions that are usually used to imitate the marine environment and to balance osmotic processes in the bacteria cells. Negligible concentration of ROS in 3% NaCl bacteria-free solutions is evident from Fig. 2A.

Additionally, Fig. 2A demonstrates that bacterial suspension produces ROS in non-radioactive solutions: concentration of ROS in the bacterial suspension (control) was about one order higher than that in 3% NaCl solution. This result is in accordance with an actual conception on continuous generation of ROS by all living organisms and cells in the metabolic redox processes (Suzen et al., 2017).

ROS content in bacteria-free 3% NaCl solution was studied in the presence of HTO. Figure 2 demonstrates that addition of HTO to the bacteria-free solution did not increase amount of ROS at three HTO radioactivities used (0.03, 4, and 500 MBq/L); the ROS content was low in all radioactive and non-radioactive bacteria-free NaCl solutions (compare Fig.2A and Fig.2B).

Low concentrations of ROS in bacteria-free HTO in the activity concentration range of 0.002–250 MBq/L were reported earlier (Selivanova et al., 2014). This paper compared ROS content in bacteria-free HTO and in solutions of $^{241}Am(NO_3)_3$, alpha-emitting radionuclide of high specific radioactivity. In contrast to tritium, a significant excess of ROS and dependence of ROS content on ^{241}Am concentration were demonstrated in bacteria-free aquatic environment (Alexandrova et al., 2011; Kudryasheva and Rozhko, 2015). The differences can be explained with different energy of radioactive decay of ^{241}Am and tritium (5637.8 and 18.6 keV (Audi et al., 2003), respectively). The

results, current and previous, show that low-intensity tritium radiation (< 500 MBq/L) cannot provide noticeable ROS production in bacteria-free water solutions under conditions of the experiment.

Addition of HTO to bacterial suspension increased amount of ROS significantly: Fig.2B demonstrates the 3-time rise of ROS content, as compared to non-radioactive bacterial suspension (control, Fig.2A). There exists evidence in the paper by Hasler (Hasler et al., 2017) that tritiated water can stimulate production of ROS in the other bacteria type, *Pseudendoclonium basilense* (bacterial strain from stagnant water bodies).

It should be paid attention that ROS contents were similar for three activity concentrations of HTO (0.03, 4, and 500 MBq/L) with the difference about four orders between the minimal and the maximal ones, Fig.2B. As evident, the effect of tritium on ROS content is similar to this on the bioluminescence of the bacteria (Fig.1).

3.3. Correlations between bioluminescence intensity and ROS content

Spearman rank correlation coefficients r were calculated between bacterial bioluminescence intensity and ROS content in non-radioactive (control) and radioactive bacterial suspensions. The r-values are presented in Table 1.

The results reveal a high positive correlations ($r > 0.7$) between bacterial bioluminescence intensity and ROS content in all bacterial environments, control and radioactive. The differences among all r values (Table 1) are statistically insignificant; so we can suppose that processes of the same probabilistic character are responsible for these correlations in all four bacterial environments, i.e. similar bacterial metabolic processes take place in non-radioactive and radiative environments.

We can conclude that activation of bacterial bioluminescence physiological function by tritium is related with ROS production in the bacterial environment. Since the rates of ROS production in bacteria-free HTO are low, the rise of the ROS production in bacterial suspensions is a result of intensification of the metabolite oxygen-dependent processes in the bacterial cells.

Hence, peculiarities of HTO effect on the luminous bacteria cells at low-dose chronic exposures are the following:

1. Low ROS content in bacteria-free aquatic environment;

2. Activation of the bacterial bioluminescence function and increase of ROS content;

3. Correlations between bioluminescence intensity and ROS content;

4. Independence of bioluminescence intensity and ROS content on activity concentration of tritium.

These peculiarities might be attributed to:

1. Low energy of tritium radioactive decay;

2. Trigger function of products of tritium decay in bacterial metabolic oxygen-dependent processes, with the bioluminescence process involved. Involvement of several cells to the initial activation by tritium;

3. Signaling role of ROS as intercellular messengers for "bystander effect" in the bacterial suspension;

4. Fixed amount of bacterial cells provided the upper limit of the bioluminescence intensity and ROS content.

4. Conclusion

We confirmed in this paper, that rates of ROS production are low in highly-diluted tritiated water. This result can be explained by low energy radioactive decay of tritium. However, exposure of marine bacteria to low-dose tritium irradiation increases ROS content in the bacterial environment considerably. We demonstrated that the rise of ROS concentration correlates with the intensification of physiological bioluminescence process in the bacteria during the bacterial lifetime. The effects can be explained by (1) "trigger" function of products of tritium decay for bacterial metabolic oxygen-dependent processes (involving bioluminescence), and (2) signaling role of ROS in "bystander effect" in the bacterial suspension. The ROS serve as intercellular messengers in the latter process.

In spite of low energy of tritium decay, its influence on living organisms in low-activity water environment might be considerable. The mechanism of tritium influence on aquatic biota can be concerned with secondary processes, i.e. production of excess amount of ROS by microorganisms due to intensification of their cellular metabolic processes. The extra content of ROS can produce either positive or negative effects on water inhabitants. Rise of ROS content should be always taken into

consideration under conditions of tritium contaminations in World Ocean, as well as in rivers, lakes, and other natural water ecosystems.

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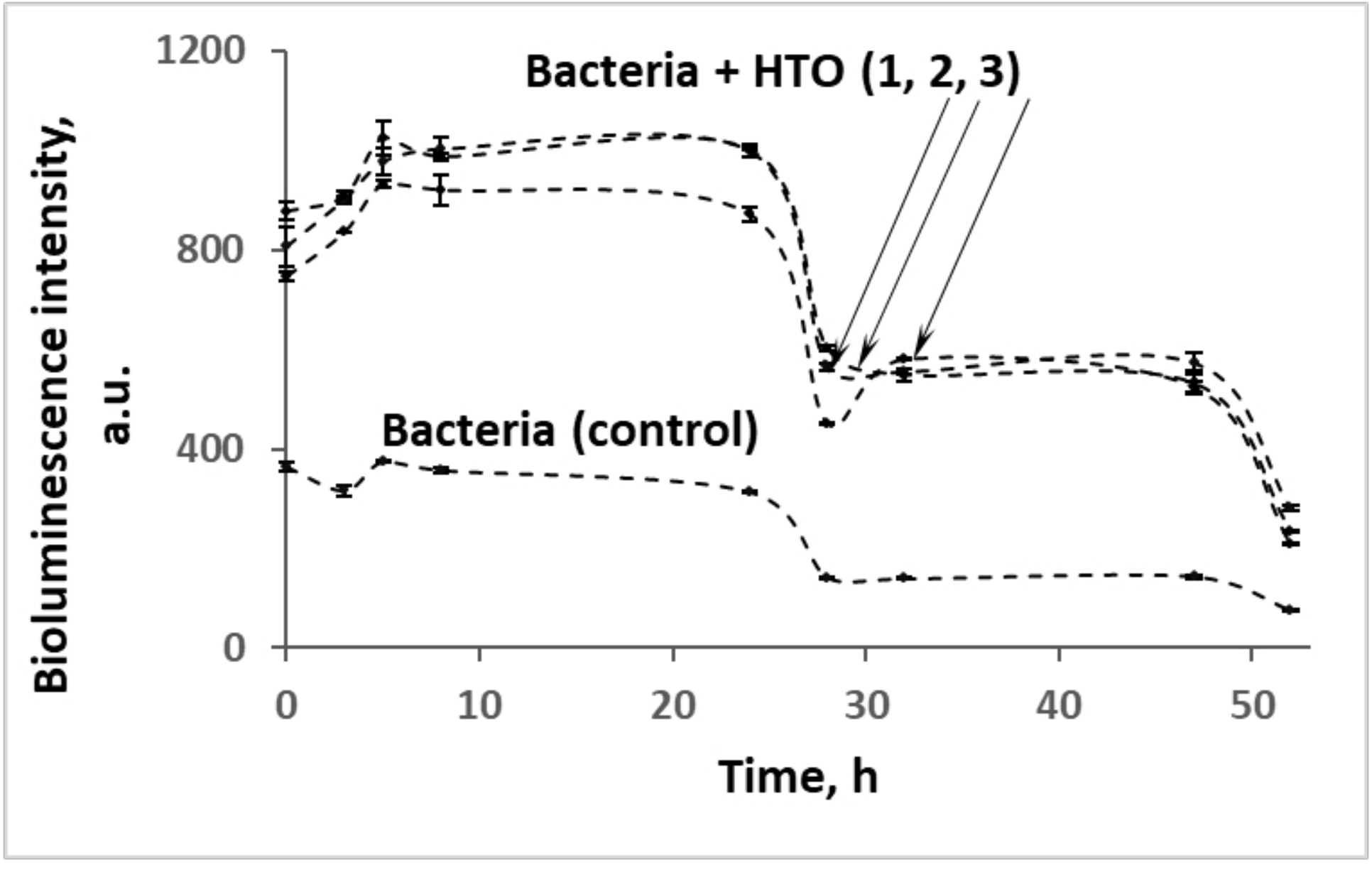
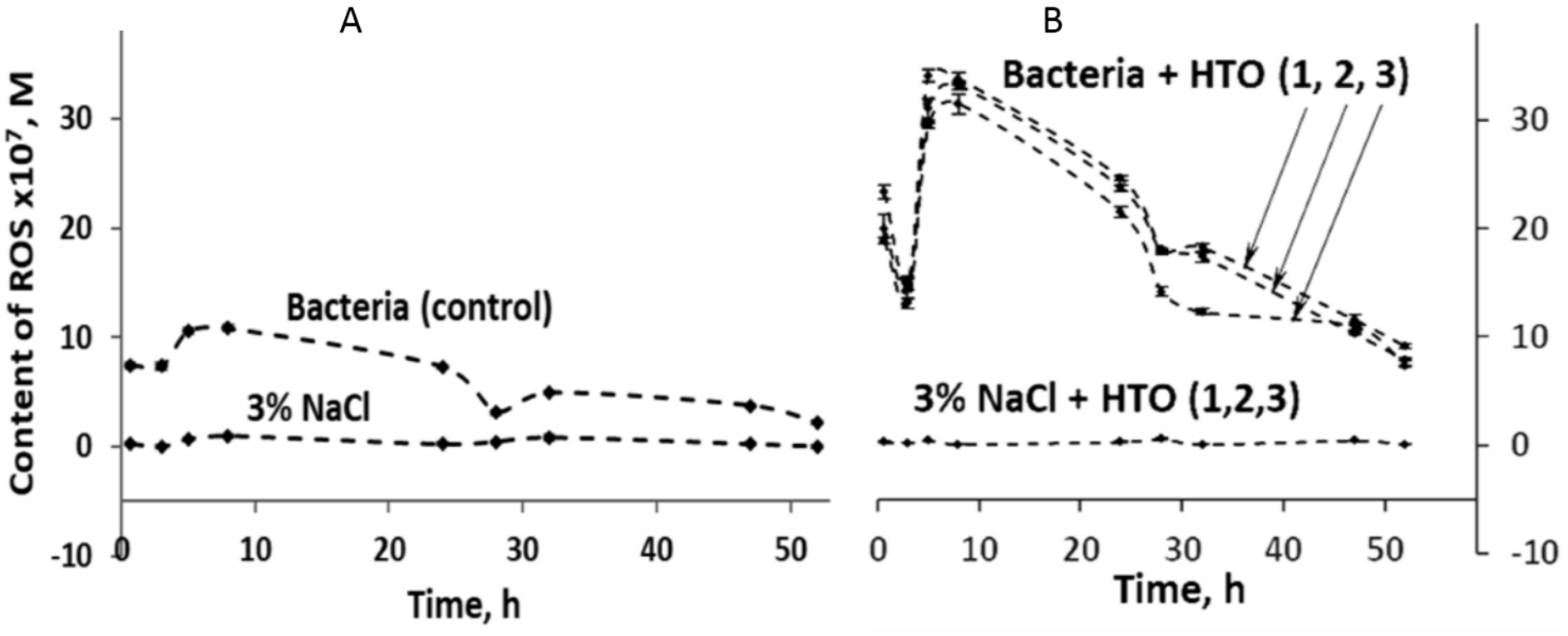
Figure captions:

Fig.1. Bacteria bioluminescence kinetics in HTO for control and radioactive samples. Activity concentrations: 1 – 0.03 MBq/L; 2 – 4 MBq/L; 3 – 500 MBq/L.

Fig.2. ROS content in: A – control bacterial suspensions and bacteria-free 3% NaCl solutions in the absence of HTO; B – bacterial suspensions and bacteria-free 3% NaCl solutions in HTO: 1– 0.03 MBq/L; 2 – 4 MBq/L; 3 – 500 MBq/L.


Bacteria + HTO (1, 2, 3)
Bacteria (control)
Bioluminescence intensity, a.u.
1200
800
400
0
0
10
20
30
40
50
Time, h


A
B
Content of ROS x10^7, M
Bacteria (control)
3% NaCl
Bacteria + HTO (1, 2, 3)
3% NaCl + HTO (1,2,3)
Time, h
Time, h
30
20
10
0
-10
0
10
20
30
40
50

r			
control	1	2	3
0,891	0.830	0.903	0.897

Table.1. Spearman correlation coefficients *r* between bacterial bioluminescence intensity and ROS content in non-radioactive (control) and radioactive (1, 2, 3) bacterial suspensions. Activity concentration of tritium: 0.03 MBq/L (1), 4 MBq/L (2). and 500 MBq/L (3).

Bystander effect via signaling function of Reactive Oxygen Species (ROS) is a possible mechanism of low dose effect of tritium on luminous marine bacteria